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Mini Review

Structural Biology and Protein Engineering of Thrombolytics

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ABSTRACT

Myocardial infarction and ischemic stroke are the most frequent causes of death or disability worldwide. Due to their ability to dissolve blood clots, the thrombolytics are frequently used for their treatment. Improving the effectiveness of thrombolytics for clinical uses is of great interest. The knowledge of the multiple roles of the endogenous thrombolytics and the fibrinolytic system grows continuously. The effects of thrombolytics on the alteration of the nervous system and the regulation of the cell migration offer promising novel uses for treating neurodegenerative disorders or targeting cancer metastasis. However, secondary activities of thrombolytics may lead to life-threatening side-effects such as intracranial bleeding and neurotoxicity. Here we provide a structural biology perspective on various thrombolytic enzymes and their key properties: (i) effectiveness of clot lysis, (ii) affinity and specificity towards fibrin, (iii) biological half-life, (iv) mechanisms of activation/inhibition, and (v) risks of side effects. This information needs to be carefully considered while establishing protein engineering strategies aiming at the development of novel thrombolytics. Current trends and perspectives are discussed, including the screening for novel enzymes and small molecules, the enhancement of fibrin specificity by protein engineering, the suppression of interactions with native receptors, liposomal encapsulation and targeted release, the application of adjuvants, and the development of improved production systems.

Contents

Abbreviations: EGF, Epidermal growth factor domain; F, Fibrin binding finger domain; K, Kringle domain; LRP1, Low-density lipoprotein receptor-related protein 1; MR, Mannose receptor; NMDAR, *N*-methyl-D-aspartate receptor; PAI-1, Inhibitor of tissue plasminogen activator; P, Proteolytic domain; Plg, Plasminogen; Plm, Plasmin; RAP, Receptor antagonist protein; SK, Streptokinase; SAK, Staphylokinase; t-PA, Tissue plasminogen activator.

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1. Introduction

1.1. Plasminogen Activators

Thrombolytics are widely used in the treatment of thrombotic diseases. The first thrombolytic drug - streptokinase - revolutionized the treatment of acute myocardial infarction [1]. Nowadays, thrombolytics are used for the treatment of *acute myocardial infarction*, *acute ischemic stroke*, *pulmonary embolism,* and other diseases. The advantages of thrombolytic therapy over mechanical clot-removal methods are cost-effectiveness, early onset of effect, the outlook for prehospital use, and availability in the developing countries with limited access to specialized centers providing mechanical clot-removal methods [2]. Thrombolytics activate the endogenous proenzyme plasminogen and convert it to the active form plasmin, which degrades fibrin and dissolves the blood clot (Fig. 1).

Study of the two endogenous plasminogen activators, *tissue plasminogen activator* (t-PA) and *urokinase*, resulted in finding their numerous roles in human pathophysiology. Many side-effects were described upon administration of t-PA into the brain, mainly *intracranial hemorrhage*, *brain edema*, *excitotoxicity*, and *neuroinflammation* [3–5]. Consideration of these side-effects is therefore critical during both treatment and development of new therapeutics. Recent studies revealed the potential of targeting the fibrinolytic system for treating neurodegenerative and psychiatric disorders [6–8]. Urokinase has a crucial role in cell migration and neoangiogenesis. Specific inhibitors of urokinase have proven to inhibit metastasis of various tumors, both *in vitro* and *in vivo* [9].

1.2. Thrombolysis

Plasminogen activators can be divided by their mode of action into two groups: (i) direct and (ii) indirect. Direct plasminogen activators are eukaryotic serine proteases which activate *plasminogen* by its cleavage at the Arg561-Val562 bond, resulting in catalytically active *plasmin*. Examples of direct plasminogen activators are t-PA [10], urokinase [11] or their variants [12]. Indirect plasminogen activators are prokaryotic proteins which bind a molecule of plasminogen and induce its conformational change in a way that it can directly convert another molecule of plasminogen to plasmin. Plasmin then cleaves fibrin and eventually dissolves the thrombus. Examples of indirect plasminogen activators are *streptokinase* and *staphylokinase*.

Plasminogen is a 791 amino acids long trypsin-like serine protease glycoprotein [13] with a molecular weight of 93 or 98 kDa, depending on the glycoform [14]. It can be glycosylated at Ser249, Asn289, Thr340, and Thr346 [15], and phosphorylated at Ser578 [16]. It consists of a PAN/apple domain, five kringle domains, a flexible linker where cleavage occurs, and a serine protease domain with a catalytic triad His603, Asp646, and Ser741 [15]. The kringle domains contain lysine binding sites which allow plasminogen to bind fibrin and other substrates containing N-terminal lysines. Both epsilon-aminocaproic acid and tranexamic acids bind to the lysine binding sites of plasminogen and plasminogen activators such as t-PA and urokinase. Hence, these acids act as competitive inhibitors and are used as antifibrinolytic drugs [17]. Plasminogen can be activated into *plasmin* by the Arg561-Val562 bond cleavage by a direct plasminogen activator [18] or via an indirect mechanism. The residues on the N-terminal side of the cleavage (1–561) form the A chain, which contains the apple domain and five kringle domains. These five domains mediate affinity to fibrin, cellular receptors and other substrates [19,20]. The A chain is linked to the B chain via disulfide bonds Cys548-Cys666 and Cys558-Cys566. The seven domains of the native form of Glu-plasminogen are in a closed activation-resistant conformation [21], which loosens up after (i) binding fibrin or (ii) removal of amino acids 1–77 by plasmin, becoming Lys-

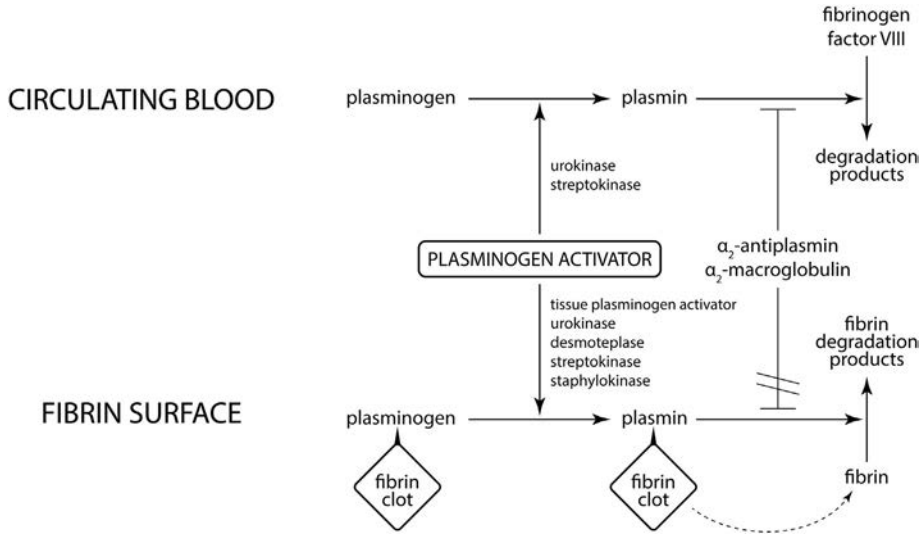

Fig. 1. A general principle of thrombolysis. Well-characterized plasminogen activators (tissue plasminogen activator, urokinase, desmoteplase, streptokinase, staphylokinase) show a unifying principle of activating plasminogen into the active form plasmin. If plasmin is bound to the surface of a fibrin clot, it digests selectively only fibrin to form soluble fibrin degradation products. This process cannot be inhibited by α_2-antiplasmin or α_2-macroglobulin because the recognition site of plasmin is sterically hindered by bound fibrin. If plasmin is generated in circulating blood, it can digest fibrinogen and factor VIII instead of fibrin. This process is rapidly inhibited by α_2-antiplasmin or α_2-macroglobulin. Fibrinogenolysis and subsequent plasminemia caused by inhibition often lead to extensive bleeding complications. As a consequence, only plasminogen activator highly selective towards fibrin-bound plasminogen can be effective in the treatment of cardiovascular diseases.

plasminogen [22]. Lys-plasminogen is less resistant to activation. The crystal structure of full-length plasminogen has been elucidated, revealing the closed conformation [23]. Full-length plasminogen promotes neoangiogenesis via extracellular matrix degradation [24]. Plasmin is cleaved by serine proteases and matrix metalloproteinases into angiostatin, a potent angiogenesis inhibitor [25]. There are two forms of angiostatin: (i) the one corresponding to domains K1–K4, and (ii) a shorter fragment of plasminogen consisting of domains K1–K3 [26]. Angiostatin inhibits angiogenesis by numerous pathways such as competing with plasmin on annexin A2/S100A10, regulating intracellular pH of endothelial cells, and interacting with other receptors on the endothelial membrane [27]. The crystal structure of the K1–K3 angiostatin has been elucidated [28].

Development of novel plasminogen activators aims to improve *thrombolytic effectivity* which is determined by the number of patients with successful recanalization of the clotted vessel. Thrombolytic effectivity is mediated by four main mechanisms: (i) rate of fibrin-specific activation of plasminogen by a plasminogen activator in the clot [29], (ii) penetration of plasminogen activator into the clot [30–32], (iii) resistance to inhibition [33–35], and (iv) clearance through the uptake by cell receptors [28–30]. Important is also *time to recanalization* in acute myocardial infarction [36,37] and volume of reperfused brain tissue in arterial ischemic stroke [38–40]. Time to recanalization is influenced by both the effectivity of the thrombolytic to degrade the blood clot and the time which elapses from the occlusion until the thrombolytic is applied [2].

Moreover, the treatment outcome is influenced by *harmful side-effects:* (i) bleeding complications, (ii) reocclusion [41,42], (iii) neurological side effects such as excitotoxicity [43,44] and damaging of the blood-brain barrier [45] leading to subsequent development of intracranial hemorrhage and brain edema. The side effects limit the usage of thrombolytics approved by the Food and Drug Administration in acute ischemic stroke by a therapeutic time window of 4.5 h from symptom onset [46]. Prolonging the therapeutic time window by avoiding the side-effects could lead to thrombolytic therapy for more patients. Given that time to therapy is a crucial predictor of the outcome, striving for less pronounced side-effects or pre-hospital therapy could lead to earlier application of thrombolytics, and therefore a better outcome [2]. *Immunogenicity* is another major concern for non-human plasminogen activators.

The most commonly clinically used thrombolytics is t-PA [12]. One of the advantages of t-PA (Table 1) over the first-generation thrombolytics is its fibrin specificity [47]. The fibrin specificity is directly connected to organization of the domains within protein molecules (Fig. 2). Non-specific thrombolytics, e.g., β-hemolytic streptococcal *streptokinase* and the second endogenous thrombolytic, *urokinase*, cause plasma fibrinogen depletion and bleeding complications (Fig. 3). The disadvantage of high fibrin specificity of

Table 1
A comparison of three generations of thrombolytics in terms of their structure and biological properties.

Thrombolytics	Domains	Protein data bank IDs	Mode of action	Fibrin affinity	Fibrin selectivity	Half-life [min]	Risk of inhibition	Risk of side-effects
First generation								
Streptokinase	α, β, γ	1BML	Indirect	None	None	10	Low	High
Urokinase	EGF, K1, P	4DVA, 2I9B	Direct	None	Low/none[a]	8	High	High
Second generation								
Tissue plasminogen activator	F, EGF, K1, K2, P	1TPG, 1TPK, 1BDA	Direct	Moderate	Moderate	4.5	High	Low
Third generation								
Desmoteplase	F, EGF, K1, P	1A5I	Direct	High	High	138	Low	Low
Staphylokinase	α	1BUI	Indirect	None	High	6	Low	Moderate

[a] Prourokinase is fibrin-selective, urokinase is not fibrin-selective.

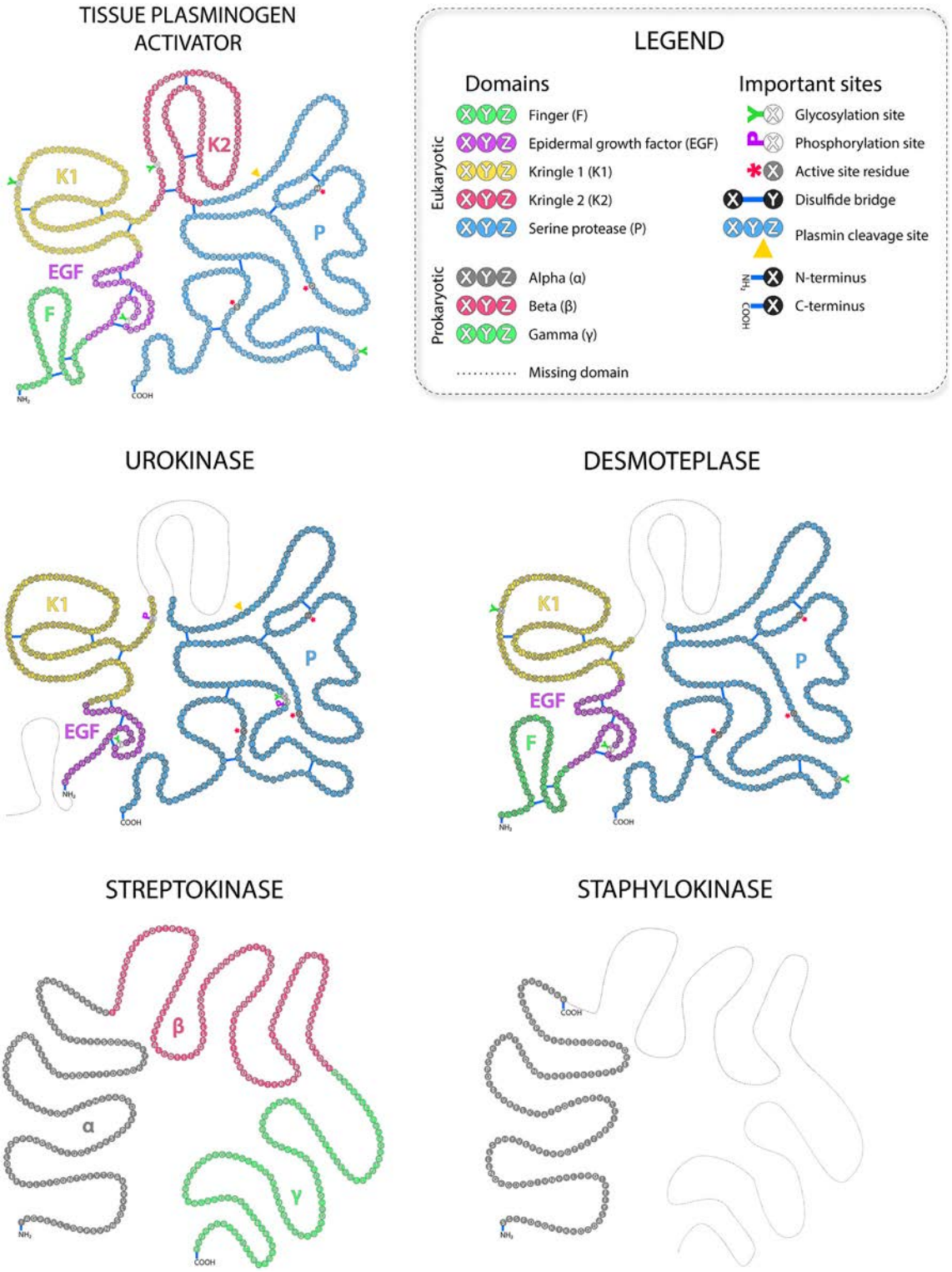

Fig. 2. Comparison of domain organization in eukaryotic and prokaryotic plasminogen activators. The corresponding domains for the eukaryotic proteins t-PA, urokinase, and desmoteplase – finger F, epidermal growth factor EGF, kringle K1, kringle K2, serine protease P – are depicted with individual colors. Glycosylation sites are marked with a green Y symbol, phosphorylation sites with a purple P symbol, catalytic residues with a red asterisk *, disulfide bridges with a blue line, and the site of the proteolytic cleavage is marked with a yellow triangle Δ. The individual domains α, β, and γ are marked with corresponding colors for prokaryotic proteins streptokinase and staphylokinase. The missing domains in homologous proteins are illustrated with a dashed line.

t-PA is front-like lysis, where most of the t-PA binds to the first micrometers of the fibrin clot. This binding hinders the penetration of t-PA inside and disables the lysis in the whole volume of the clot, causing non-optimal effectivity. This uneven lysis pattern can lead to the formation of smaller clots which are released from the blood vessel wall and can cause reocclusion [30,32,48]. The third generation of thrombolytics are: (i) derivatives of t-PA engineered for enhanced half-life, penetration into a clot, fibrin specificity and

resistance to inhibition, e.g., *reteplase*, *tenecteplase*, *duteplase*, *monteplase*, *lanoteplase*, *pamiteplase*, *amediplase* [12], (ii) *desmoteplase* from the vampire bat saliva, and (iii) fibrin-specific prokaryotic *staphylokinase*.

2. Tissue Plasminogen Activator

2.1. Biological Function

Besides fibrin-specific thrombolysis, t-PA also has many roles in the brain (Fig. 4). A high concentration of t-PA in the bloodstream during therapeutic thrombolysis can result in deleterious side-effects. t-PA is a modulator of cerebral blood flow and blood-brain barrier permeability in response to neuronal activity and facilitates memory formation and response to brain injury [3,29,45,49–53]. Both t-PA and the t-PA-PAI-1 complex have effects on blood vessel tone. The t-PA-PAI-1 complex induces vasodilation whereas t-PA alone induces vasoconstriction. Vasodilation can have beneficial effects of better penetration into the blood clot, which could be a downside of tenecteplase [54].

2.2. Molecular Structure

T-PA is a 527 amino acids long glycoprotein belonging to the trypsin-like serine protease family (Fig. 5). It exists in two glycoforms: type 1 and type 2 t-PA with a molecular weight of 66 or 63 kDa, respectively. Type 1 has oligosaccharides bound at both Asn448 and Asn184, while type 2 is glycosylated at Asn448 only [55]. T-PA carries 17 disulfide bridges which make it hard to express in prokaryotic systems and many studies are focused on improvement of its production. T-PA is composed of five domains [56]: (i) the fibronectin-like finger F domain (residues 1–49), (ii) the epidermal growth factor EGF domain (50–87), (iii) kringle K1 domain (88–175), iv) kringle K2 domain (176–256), and v) the serine protease P domain (257–527, Fig. 6).

The domain F contributes to about 80 % of the affinity to fibrin [57]. It also binds to annexin A2/S100A10 located on the cell surface [58] and is involved in the low-density lipoprotein receptor-related protein 1 (LRP1) binding together with the domains EGF and K1 [59]. The EGF domain activates the EGF receptor [60] and is fucosylated on Thr61 [61]. The domain K1 binds to the mannose receptor (MR) expressed in liver endothelial and Kupffer cells by high-mannose oligosaccharide linked

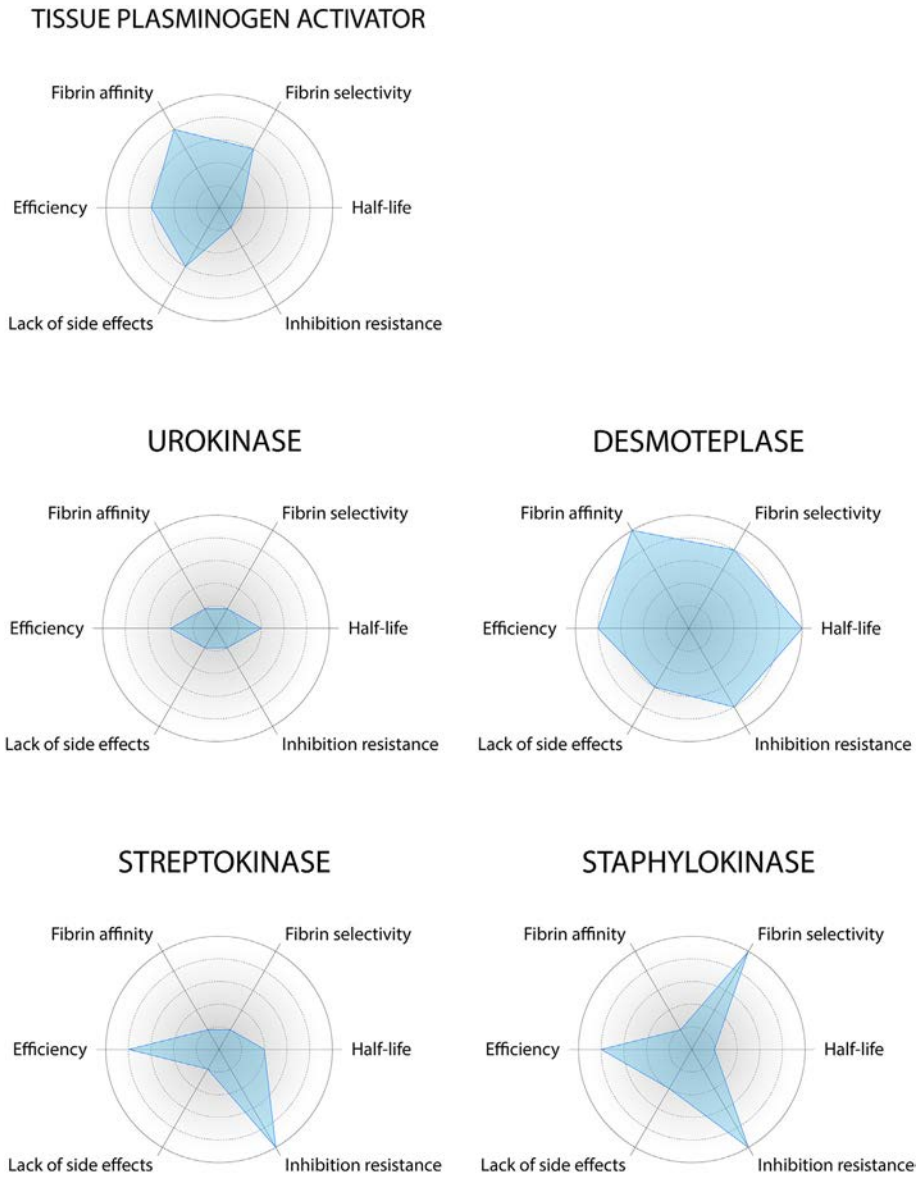

Fig. 3. Comparison of key properties of eukaryotic and prokaryotic plasminogen activators. The size of the blue area quantifies the overall potential for clinical use of the protein (large area = high potential). The radial plots are based on the values published in the scientific articles cited throughout this review article.

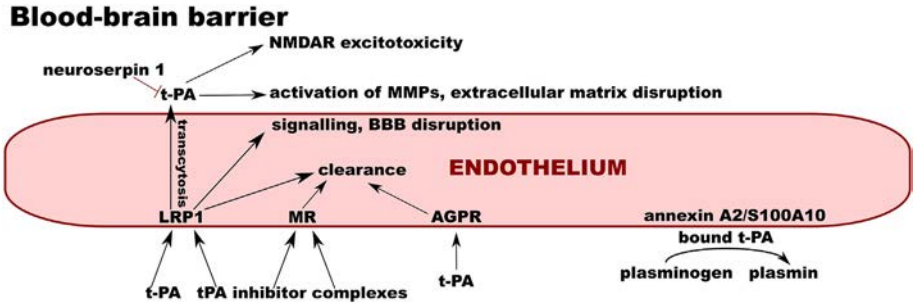

Fig. 4. Interactions of t-PA on the blood-brain barrier. On the endothelial cell surface, tissue plasminogen activator (t-PA) binds annexin A2/S100A10. This leads to plasminogen activation on the cell surface. The free form, as well as inhibitor complexes of t-PA, are endocytosed by asialoglycoprotein receptor (AGPR), mannose receptor (MR), and low-density lipoprotein receptor-related protein 1 (LRP1), leading to their clearance. T-PA can cross the intact blood-brain barrier (BBB) via LRP1-dependent transcytosis. In the brain parenchyma, it can activate matrix metalloproteinases and interact with the N-methyl-D-aspartate receptor (NMDAR). These effects are inhibited by neuroserpin 1, the inhibitor of tPA in the brain parenchyma.

to Asn117 [62]. The domain K2 plays a crucial role in the stimulation of proteolytic activity by fibrin [63] and by other lysine-containing substrates [64]. The domain K2 contains a negatively charged lysine binding site around Asp236 [65] which binds fibrin, amyloid beta aggregates [66], *N*-methyl-D-aspartate receptor (NMDAR) [67], and others via non-specific interaction with C-terminal and intra-chain lysines [68]. The domain P is catalytically active and carries a catalytic triad of His322, Asp371, and Ser478 [69].

2.3. Mechanism of Activation and Specificity

Most serine proteases are expressed in an inactive, single-chain form and must be cleaved to the two-chain active form. T-PA is cleaved between Arg275-Ile276 by plasmin [70] into N-terminal "A chain" and the C-terminal "B-chain" which are connected by a single disulfide bond between Cys264-Cys395 [71]. While other serine proteases are more than 10^7 times less active in the single-chain form, t-PA has low zymogenicity with only 5–10-fold increase in activity upon cleavage [72]. The catalytic activity of t-PA is regulated by a dynamic conformational equilibrium of the activation domain [39,73,74] restricting the binding of plasminogen in the inactive states. Activation domain contains the catalytic triad, an oxyanion hole and an S1 specificity pocket [69]. Active conformations are favored when a salt bridge is formed between Asp477 and the N-terminal of the B-chain of two-chain t-PA. Low zymogenicity of single-chain t-PA is caused by substitute salt bridge of Lys429 and Asp477, which leads to an active conformation.

T-PA has a general trypsin-like specificity, preferentially cleaving peptide bonds after Arg and a small or a hydrophobic residue. The

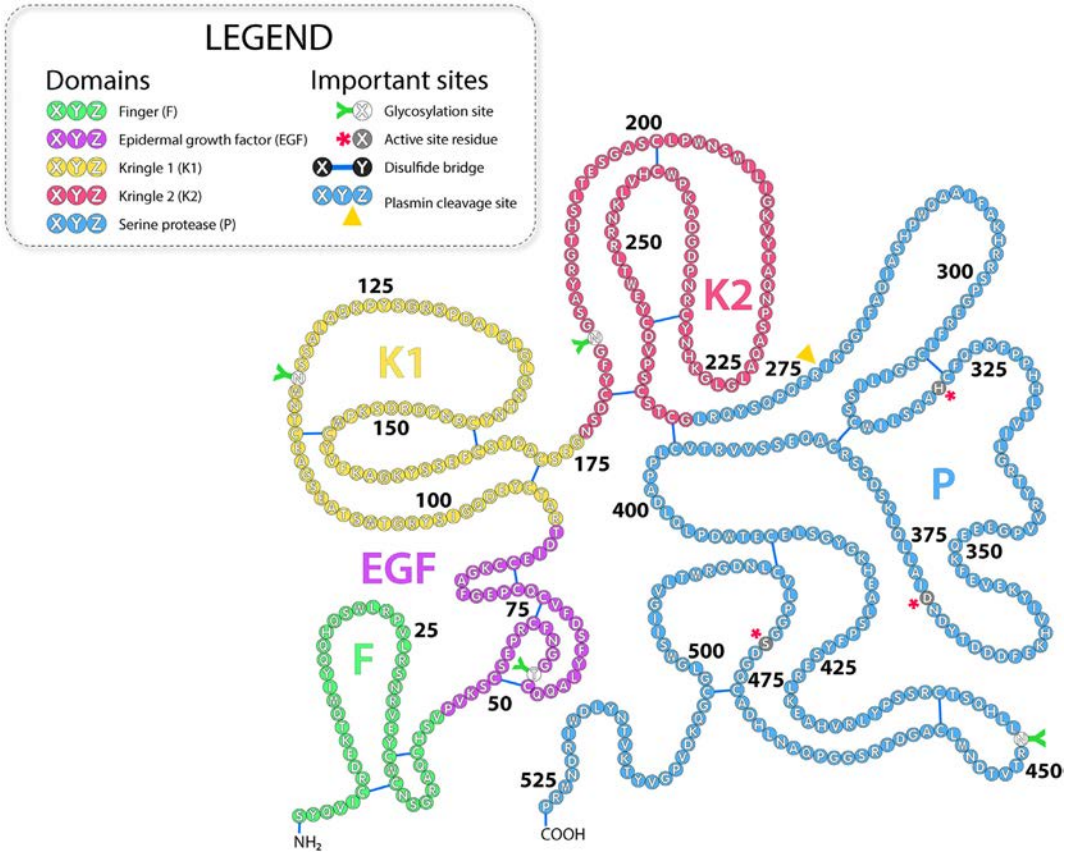

Fig. 5. The primary structure of tissue plasminogen activator. Individual domains – finger (F), epidermal growth factor (EGF), kringle 1 (K1), kringle 2 (K2), serine protease (P) , important residues, cysteine-bridges, cleavage sites and sites of post-translational modification are highlighted as stated in the legend.

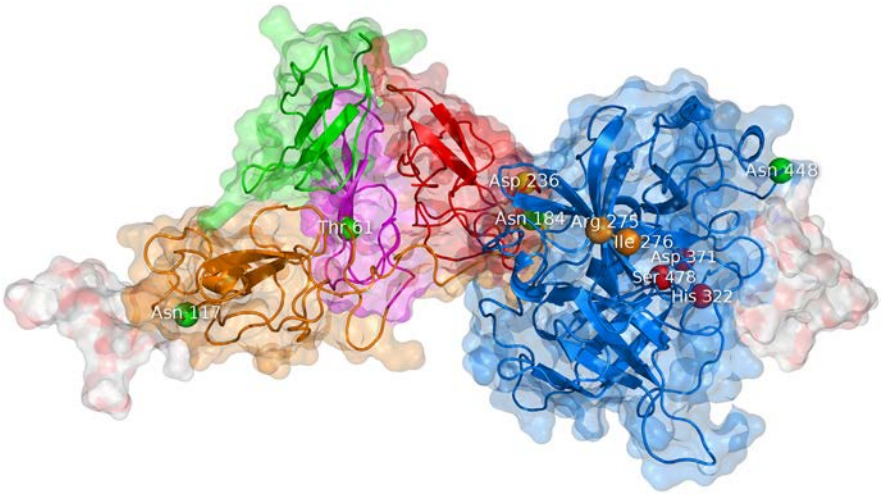

Fig. 6. Theoretical model of the tertiary structure of tissue plasminogen activator. The visualization is based on the model kindly provided by Ashish and coworkers [78]. T-PA is composed of five domains: finger F (green), epidermal growth factor EGF (purple), kringle K1 (orange), kringle K2 (red), and protease P (blue). The molecule contains four glycosylation sites labeled as green beads: Thr61, Asn117, Asn184, and Asn448. The molecule also has complex oligosaccharides attached to Asn117 and Asn448, which are shown as white surface. The K2 domain contains a lysine binding site at Asp236 (yellow bead). The P domain can be cleaved into two chains in between Arg275 and Ile276 (orange beads). The catalytic triad of the P domain is His322, Asp371, and Ser478 (red beads).

plasminogen cleavage site is a Cys-Pro-Gly-Arg-Val-Val-Gly-Gly-Cys cyclised by the flanking cysteines. The active site cleft of t-PA is specifically well suited for cleaving it since linear peptides mimicking the plasminogen cleavage site are cleaved 10^4 times less efficiently than plasminogen [75]. The adaptation to the cyclic sequence in plasminogen is determined by loops around the active site [72] and the specificity for plasminogen is enhanced by a hydrophobic exosite at the residues 420–423 [76].

2.4. Binding and Stimulation by Fibrin

T-PA variants with: (i) uncleavable single-chain form [67,77], (ii) restored zymogen triad [77], or (iii) the single-chain form salt bridge disrupted [70,72], are stimulated by fibrin to a similar activity as the wild type t-PA. The property of fibrin stimulation limits systemic fibrinogen depletion. All five domains of t-PA account for fibrin binding, with the strongest effect of the F domain followed by the K2 domain. The stimulation of activity is mediated by the heavy-chain domains via co-localization of t-PA and plasminogen on fibrin in a productive orientation and by increasing the catalytic rate of t-PA.

The glycosylation at Asn184 insulates contacts between the F, K2, and P domains, in the less active type 1 [78]. The orientation of the F, E, K2, and P domains, towards each other is mediated by the domain K1, which explains the deleterious effect of deletion of any domain on activity and stimulation by fibrin [79]. Binding of the penta-L-lysine peptide between the K2 and P domains stimulated the activity by increasing the number of inter-domain interactions and reducing protein dynamics [78]. Domain deletion studies have reported inconsistent results, some indicating the K1 domain is not significantly involved in binding [57,80], whereas others suggested that one kringle domain, be it K1 or K2, can facilitate fibrin binding [63]. The F domain contains patch of charged residues Arg7, Asp8, Glu9 and Lys10, while the P domain (Fig. 7) contains a fibrin binding patch of the residues His432, Arg434, Asp460, and Arg462 [69,81].

T-PA's high selectivity for fibrin over fibrinogen [82] is mediated by cryptic sites present in fibrin [83]. Cross-linked αC domain fragments

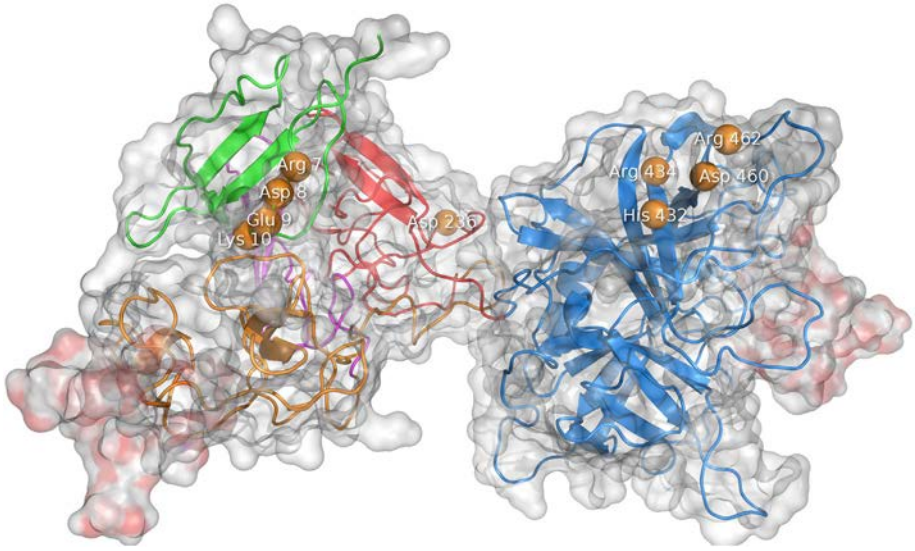

Fig. 7. The fibrin binding sites of tissue plasminogen activator. The residues interacting with fibrin are shown as orange beads. Two charged patches are involved in binding of fibrin: (i) Arg7–Lys10 in the t-PA's finger domain and (ii) residues His432, Arg434, Asp460, and Arg462 in the serine protease domain. The lysine binding site at Asp236 binds both C-terminal and intra-chain lysines of partially degraded fibrin.

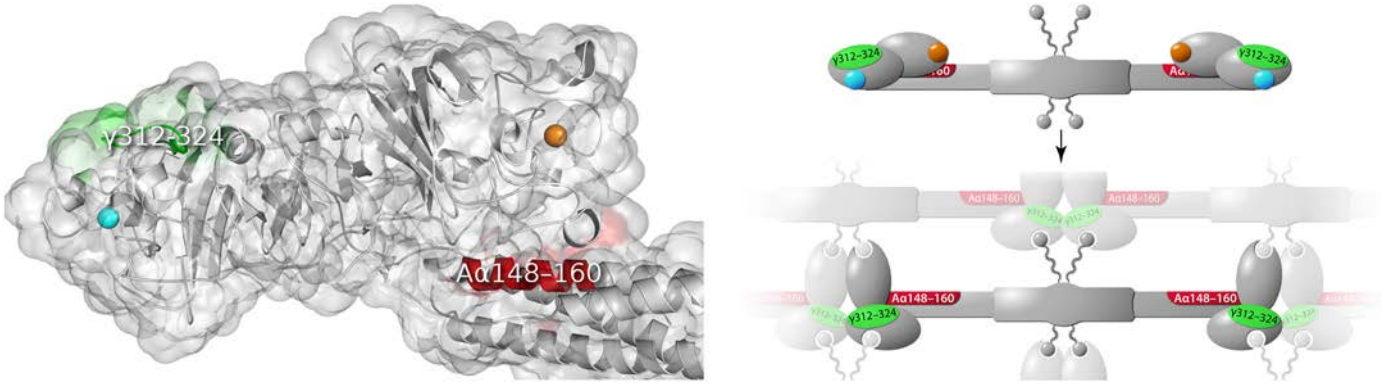

Fig. 8. Tertiary and quaternary structure of fibrin. (*Left panel*) The C-terminal part of fibrinogen (PDB ID 3GHG). The α chain of fibrinogen contains a site which can bind either t-PA or plasminogen at the residues Aα148–160 (red). Residues γ312–324 form a site which binds t-PA (green). The sites are situated very close to the A-hole (blue bead) and B-hole (orange bead) which in fibrin polymerization bind the A- and B-knobs, respectively. (*Right panel*) The t-PA and plasminogen binding sites, and the A- and B-hole are highlighted as in panel A. Upon polymerization, the A knobs interact with the A holes, the B knobs with the B holes, and the globular C-terminal parts of the α and β chain get spatially reoriented, exposing the buried Aα148-160 site.

Aα392–610 contain high-affinity binding sites for t-PA and plasminogen [84]. Their binding is lysine-dependent which suggests the domain K2 binds these sites. A low-affinity binding site on fibrin (γ312–324, Fig. 8) is positioned next to the A knob binding cavity [85]. When A knob binds, the β globular domain is pulled away and the buried binding site which can bind of t-PA or plasminogen (Aα148–160) is exposed [86,87]. Similarly, binding of B knobs also makes the Aα148–160 site accessible whereas synthetic B knobs showed fibrinolysis inhibition [88]. Limited digestion by plasmin reveals new C-terminal lysines which bind t-PA and plasminogen, resulting in stimulation of fibrinolysis [89,90].

2.5. Inhibition

T-PA is irreversibly inhibited by proteins of the serpin family: PAI-1, PAI-2, protease-nexin-1, and neuroserpin [91–94]. These inhibitors bind by their reaction center loop into the active site of t-PA [94]. T-PA cleaves the bond of its major inhibitor PAI-1 and remains covalently bound by the catalytic serine. The interaction with PAI-1 (Fig. 9) is determined by the positively charged loop 296–302 and the residue Arg304 [95,96]. The 296–302 tetra-alanine substitution in tenecteplase [97] or charge-reversing mutations in this region [98] can substantially decrease the inhibition. Other strongly interacting residues representing inhibition hot-spots are Gln325, Glu326, Asp365, and Tyr368. Especially, Tyr368 protrudes to the active site cleft of t-PA and restricts the S2 specificity pocket which causes resistance to most canonical serine protease inhibitors [69]. Tyr368Leu mutation improves the resistance of t-PA to PAI-1 without compromising the activity with plasminogen [99]. Mutation Ala419Tyr introduced into the hydrophobic pocket of t-PA formed by Ala419, Gln475 and Gly501 [96] increased PAI-1 resistance 30-fold and plasminogen activation 5-fold [100].

2.6. Interaction with Receptors and Clearance

Interactions of t-PA with receptors mediate many biological functions (Fig. 10). The most important is binding to liver cell receptors (LRP1), which are responsible for the short half-life of only 4.5 min [101]. LRP1 plays a major role in clearance, followed by MR [62], galectin [102], and potentially by the asialoglycoprotein receptor [55]. Gp330 is highly homologous to LRP1 and could be responsible for the clearance of t-PA in the kidney [103,104]. The binding sites for both free t-PA and the t-PA-PAI-1 complex on LRP1 are on complement repeats of cluster 2 of LRP1 [105]. Binding can be inhibited by the receptor antagonist protein (RAP), which inhibits the binding both by competing for the part of the binding site and allosterically [103,104,106]. The ligand-binding mechanism of LRP1 employs aspartates around Ca^{2+} ions [101,107–110] which bind exposed lysines. In t-PA and desmoteplase [111], the LRP1 binding sites could be present in the F, E, and K1 domains [58,59,112]. Tyr67Gln mutation impairs binding via an extra oligosaccharide inserted, which hinders the interaction with LRP1 [113]. The effects of LRP1 interaction with t-PA mediate pleiotropic roles of t-PA in neurophysiology [29,49], but also contribute to neurological side-effects of therapeutically used t-PA.

All these effects on the nervous system are more pronounced when t-PA is used in the treatment of acute ischemic stroke. The MR is proven to bind the high-mannose oligosaccharide attached to Asn117. Deletion of K2 reduces NMDAR-mediated neurotoxicity [114] as well as mutations in the positively charged loop (296–299) [115]. On the other hand, proteolytic activity is not necessary for NMDAR activation so the

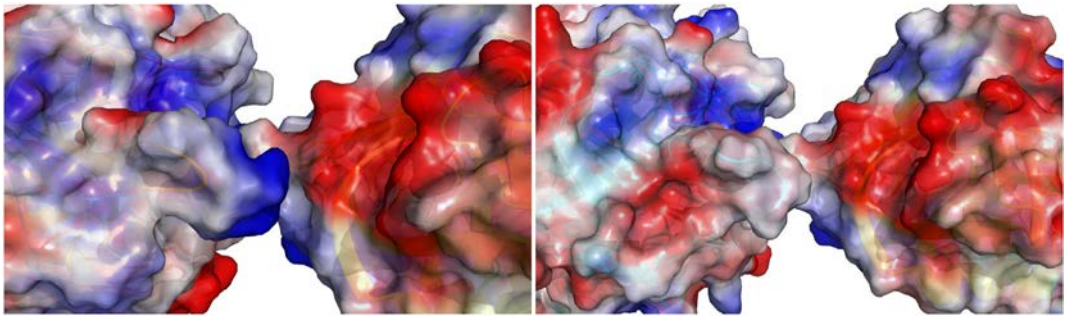

Fig. 9. Interactions of t-PA and tenecteplase with the plasminogen activator inhibitor-1. (*Left panel*) The interface between t-PA (left) and the plasminogen activator inhibitor-1 (right) with their surface colored according to the surface charge (PDB ID 5BRR). T-PA contains a positively charged loop of residues 296–302 which fits into a negatively charged cleft on PAI-1. (*Right panel*) The catalytic domain of tenecteplase (left) carries the 296–299 tetra-alanine substitution which weakens its interaction with PAI-1 (right).

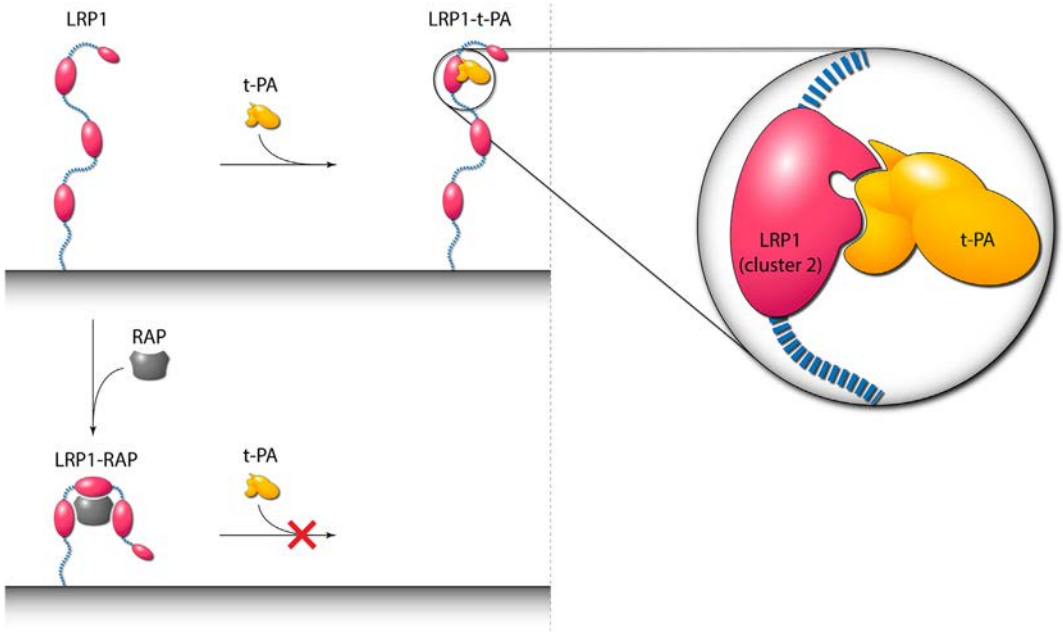

Fig. 10. Schematic representation of binding of t-PA to low-density lipoprotein receptor-related protein 1 (LRP1) and binding of receptor-associated protein (RAP). *(Left panel)* Low-density lipoprotein receptor-related protein 1 (LRP1) binds to t-PA via cluster 2 (red oval) of its structure. In contrast, receptor-associated protein (RAP) possesses three binding domains, binds three clusters of LRP1 simultaneously and induces conformational changes that prevent the binding of t-PA. RAP does not compete for the same binding site but allosterically makes the site inaccessible for t-PA binding. (*Right panel*) A close-up view of the LRP1-t-PA complex. The binding of t-PA to LRP1 is enhanced by an avidity effect of two separate binding sites of t-PA that interact with distinct complement repeats of cluster 2 of LRP1.

specific interaction remains unclear [44,115]. Desmoteplase does not contain a lysine binding site and is not neurotoxic [116]. Interestingly, only the single-chain form of t-PA activates NMDAR [43]. Since t-PA interacts with Annexin A2/S100A10 via C-terminal lysines, the lysine binding site on K2 domain is likely the determinant of the binding. Annexin A2/S100A10 also binds plasminogen at the site of the cytoplasmic membrane and is a source of plasminogen activation on the cell surface [117]. Binding to endothelial cells by t-PA's kringle domains has been shown to inhibit neoangiogenesis [118–120]. The binding of t-PA to the epidermal growth factor receptor protects neurons in ischemic conditions [60]. Binding to annexin A2/S100A10 tetramer on the surface of vascular endothelial cells enhances the activation of plasminogen on the cell surface and activates nuclear factor κB involved in immune response [121–123]. Both of these effects could cause problems in thrombolytic therapy: (i) reocclusion complications and (ii) inflammation.

2.7. Protein Engineering

One aim of protein engineering of t-PA was to increase its half-life. A deletion mutant reteplase retains only the K2 and P domains [124] and has the tetra-alanine substitution. Half-life is thus enhanced by the elimination of binding to the MR, decreased binding to LRP1 and resistance to PAI-1. Tenecteplase also contains the tetra-alanine in addition to Thr103Asn which introduces a complex oligosaccharide, causing steric hindrance of clearance receptors, and Asn117Gln eliminating MR recognition [125]. The tetra-alanine also increases fibrin specificity [126]. Monteplase with Cys84Ser in the EGF domain has a half-life of 23 min [128]. Duteplase is a Met245Val mutant produced exclusively as a two-chain form and possesses slower clearance rate than single chain t-PA [127]. Lanoteplase has a prolonged half-life to 37–45 min [128] due to the absence of a large part of the F domain, whole EGF domain and the Asn117Gln substitution [129]. Pamiteplase is a K1 domain deletion mutant with a half-life of 30–47 min. It also contains the Arg275Glu mutation which renders it uncleavable to the two-chain form [130,131]. Amediplase is a chimeric protein composed of the first 3 residues of the F domain and the K2 domain of t-PA, combined with residues 159–411 of the P domain of urokinase. The lower fibrin affinity enables better penetration into the clot. It has a half-life of 30 min [12], likely because of the absence of F, EGF, and K1 domains. A variant consisting of the GHRP peptide mimicking the B knob of fibrin, K2, and P domain has an increased half-life as well as improved binding to fibrin compared to reteplase [128]. Arg275Ser mutation is preventing the conversion to the two-chain form and thus NMDAR-mediated neurotoxicity. The variant also has the lysine binding site in K2 defunct via the Trp254Arg mutation [114]. Higher fibrin binding and fibrin stimulation were achieved by substituting the finger domain of t-PA by that of desmoteplase and deleting the K2 domain [132].

3. Urokinase

3.1. Biological Function

Urokinase accelerates thrombolysis initiated by t-PA and was tested as a synergistic therapy [48,133–136]. The implications of urokinase in cancer make it a target for the development of urokinase-specific inhibitors [137] or disrupting interactions with its receptors [138,139]. Urokinase is primarily associated with plasminogen activation on the cell surface and is involved in neoangiogenesis [140], degradation of the extracellular matrix by plasmin [141], and activation of matrix metalloproteinases [142,143]. These mechanisms confer the roles of urokinase in cell migration [144], tumor metastasis [145], cell adhesion [146], and proliferation of various cancer types [147,148].

3.2. Molecular Structure

Urokinase is a 411 amino acids long protein with a molecular weight of 54 kDa [71], having a 40 % identity with t-PA [149]. It consists of the EGF domain (1–49), a K domain (50–131) lacking a lysine binding site [150], a flexible linker (132–158), and a serine protease domain P (159–411) with the catalytic triad of His204, Asp255, and Ser356 (Fig. 11). Phosphorylations on Ser138 and Ser303 contribute to the signaling through the urokinase receptor [151]. Urokinase is fucosylated on Thr18 [152] and N-glycosylated on Asn304 [153]. The enzyme is secreted as prourokinase, which is cleaved by plasmin and other proteases

at Lys158-Ile159 to a two-chain form called high molecular weight urokinase [154]. Additional cleavage at Lys135-Lys136 generates the N-terminal EGF-K fragment and low molecular weight urokinase.

3.3. Mechanism of Activation and Specificity

The conversion to two-chain form increases inter-domain flexibility [155] which enables to interact more efficiently with its substrates and improves the activity of urokinase about 100 times [156]. Cofactors shift the equilibrium towards the formation of an active catalytic site [157] or could improve the interaction of the new Ile159 N-terminus with Asp370, analogically to the activation of t-PA [158,159].

3.4. Binding and Stimulation by Fibrin

Prourokinase cleaves relaxed plasminogen bound to fibrin [160]. Interestingly, prourokinase is stimulated only by fibrin fragment E, but not fragment D, which could be due to different conformations adapted by plasminogen when bound there. Mutation of Pro309 can make urokinase stimulated by both fragments E and DD [161]. Two-chain form urokinase is not fibrin-selective and causes systemic fibrinogen depletion.

3.5. Inhibition

Urokinase is inhibited by PAI-1, PAI-2, protease-nexin [162], and thrombin [163]. As in t-PA, the positively charged loop around His200 contributes significantly to the recognition by serpins [164]. The aim of understanding the specific inhibition of urokinase has led to crystallographic and inhibitor screening studies [164–167].

3.6. Interaction with Receptors and Clearance

Urokinase's half-life of 8 min in the human bloodstream is mediated by the receptor LRP1 and asialoglycoprotein [168]. LRP1 internalizes either free urokinase or the trimeric complex with PAI-1/PAI-2 and the urokinase receptor. The binding to LRP1 is probably mediated by domains EGF and K [169–172]. Plasminogen activation on the cell surface is mediated by Ω loop of the EGF domain of urokinase binding to the urokinase receptor [173–176]. When bound to the urokinase receptor, urokinase regulates cell adhesion by cleavage of vitronectin [146]. The modulation of chemotaxis via interaction with integrins is probably related to the presence of the flexible linker [177,178].

3.7. Protein Engineering

Chimeric combinations of t-PA and urokinase domains possess poor fibrin affinity [179]. Amediplase has poor fibrin affinity due to the interaction of the P domain from urokinase with both K domains from t-PA. On the other hand, it has better penetration into the clot and a longer half-life [12]. The mutation Lys300His has 10 times lower amidolytic activity in the single-chain form and 2 times higher in the two-chain form [180,181].

4. Streptokinase

4.1. Biological Function

Streptokinase is an exocellular plasminogen activator of the prokaryotic origin first described in 1933 [182]. It is naturally produced by the strains of β-hemolytic streptococci which utilize it for overcoming the host's defensive fibrin barrier and for promoting bacterial metastasis and colonization [183,184]. There are several streptokinases from different streptococci which vary in their structure, but the only variant of streptokinase currently used as a thrombolytic agent originates from the group C streptococci (streptokinase SK-H46A from *Streptococcus equisimilis* strain H46A) and lacks a significant stimulation by fibrin [185,186]. Despite its moderate half-life *in vivo* (approximately 10 min) and significantly lower cost, the largest disadvantages are the lack of fibrin specificity and immunogenicity due to its bacterial origin [47,187–189].

4.2. Molecular Structure

The molecule of streptokinase consists of 414 amino acids and the biological unit forms the monomeric protein with the molecular weight of 47 kDa [190,191]. The isoelectric point is 4.7, while the pH optimum is between 7.3 and 7.6 [187,191]. Nuclear magnetic resonance and circular dichroism studies [152,192] alongside with the crystallographic analysis of the microplasmin-streptokinase complex [193] showed that streptokinase contains three β-grasp domains – α, β, and γ (Fig. 12). The domain α (residues 1–150) mainly mediates the formation of the plasminogen active conformation within the streptokinase-plasminogen complex [194–200] and is important for the substrate recognition [194,197,198,201–203]. The domain β (residues 151–287) is responsible for high-affinity binding during the activation of the plasminogen "partner" [197,204–206] but it also facilitates the

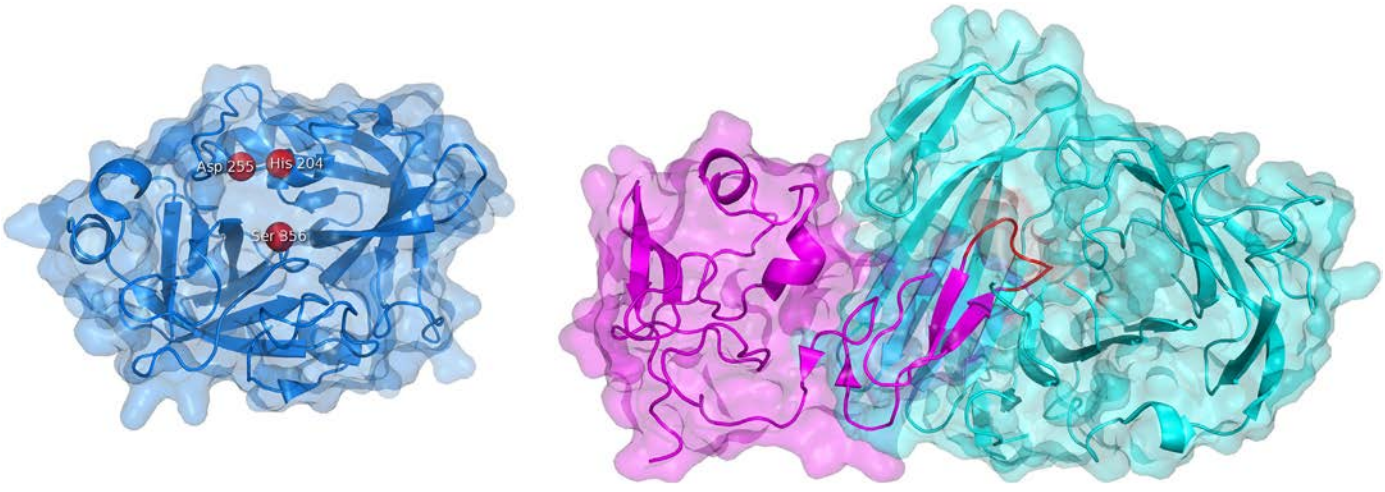

Fig. 11. The catalytic serine protease domain and epidermal growth factor domains of urokinase complexed with the urokinase receptor. (*Left panel*) The serine protease domain of urokinase (PDB ID 4DVA) with its triad of His204, Asp255, and Ser356 shown as red beads. (*Right panel*) The EGF domain of urokinase (PDB ID 2I9B) is shown as violet surface and the urokinase receptor as cyan surface. The omega loop of the amino terminal fragment which binds the urokinase receptor is highlighted in red.

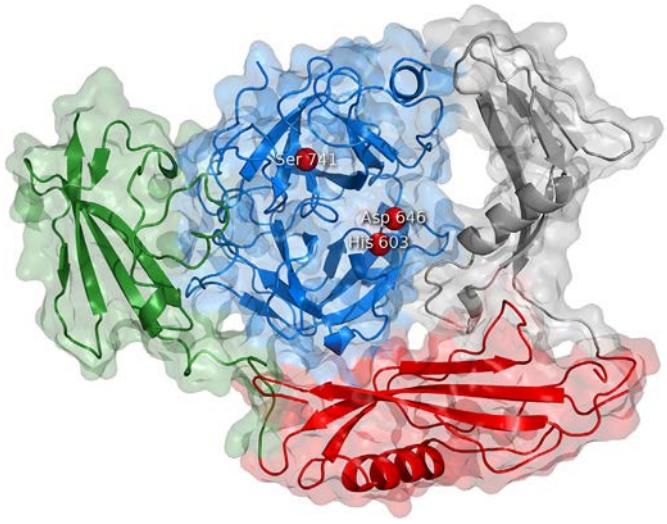

Fig. 12. The binary complex of streptokinase with bound plasmin. The complex of streptokinase and the catalytic domain of plasmin (blue) (PDB ID 1BML). Streptokinase contains three β-grasp domains embracing the plasmin catalytic domain: domain α (gray), domain β (red), and domain γ (green). The catalytic triad of plasmin His603, Asp646, and Ser741 are shown as red beads.

plasminogen "substrate" binding and processing [207–211]. Finally, the domain γ (residues 288–414) is involved in stabilizing the streptokinase-plasminogen complex and in inducing its proteolytic activity [193,197,201,204,208,212–214]. Although having one or two major functions, each domain participates in all the steps of plasminogen activation due to the high level of cooperativity [206,215,216].

4.3. Mechanism of Activation and Specificity

Compared to human-derived plasminogen activators, streptokinase represents a thrombolytic agent with an indirect mechanism of plasminogen activation (Fig. 13), i.e., it does not possess enzymatic activity. The activation process occurs in three fundamental steps including (i) formation of an equimolar (1:1) complex with the molecule of plasminogen ("partner molecule"), (ii) induced intramolecular rearrangement of the complex generating an enzymatically active structure, and (iii) amidolytic conversion of other free plasminogen molecules ("substrate molecules") to plasmin by the active streptokinase-plasminogen complex [201,217–219].

The detailed mechanism of all these steps was intensively studied and the current understanding assumes the complex behavior of all the participating structures as follows:

1. The β domain of streptokinase interacts with the lysine binding site of the kringle 5 domain of the "partner" plasminogen molecule which, originally closed, becomes more extended and allows all the three domains of streptokinase to associate with the catalytic domain of plasminogen [197,206,220,221]. Affinity towards plasminogen during the initial step is further enhanced by the C-terminal Lys414 which interacts with the plasminogen's kringle 4 domain [214,222,223].
2. Within the formed encounter complex, the N-terminal amino group of Ile1 residue of streptokinase forms a salt bridge with Asp740 of plasminogen [195,199,200,207] and simultaneously, additional salt bridges between the γ domain of streptokinase and the catalytic domain of plasminogen are created [201,212]. These interactions cause conformational changes, open the active site of plasminogen and make the overall complex enzymatically active, forming so-called "virgin enzyme", marked as SK-Plg* in Fig. 13 [197,201,217,218].
3. The conformation of the active complex allows binding of another molecule of plasminogen ("substrate") resulting in a transient ternary intermediate [201,211]. The "substrate" molecule is bound predominantly by the α domain [197,201–203] while the 250-loop of the streptokinase's β domain (residues Arg253, Lys256, and Lys257) interacts with the kringle 5 domain of the plasminogen substrate via its lysine binding site [207,208,210,211,220].
4. Finally, the Arg561-Val562 peptide bond of the "substrate" molecule is hydrolytically cleaved by the "partner's" catalytic triad His603, Asp646, and Ser741, and the final molecule of active plasmin is formed and released [201,217,219]. Regions 88–97, 164–186, and 314–342 of streptokinase were reported to be important for the plasminogen substrate processing during this final step of activation [208,209,213,215,224].

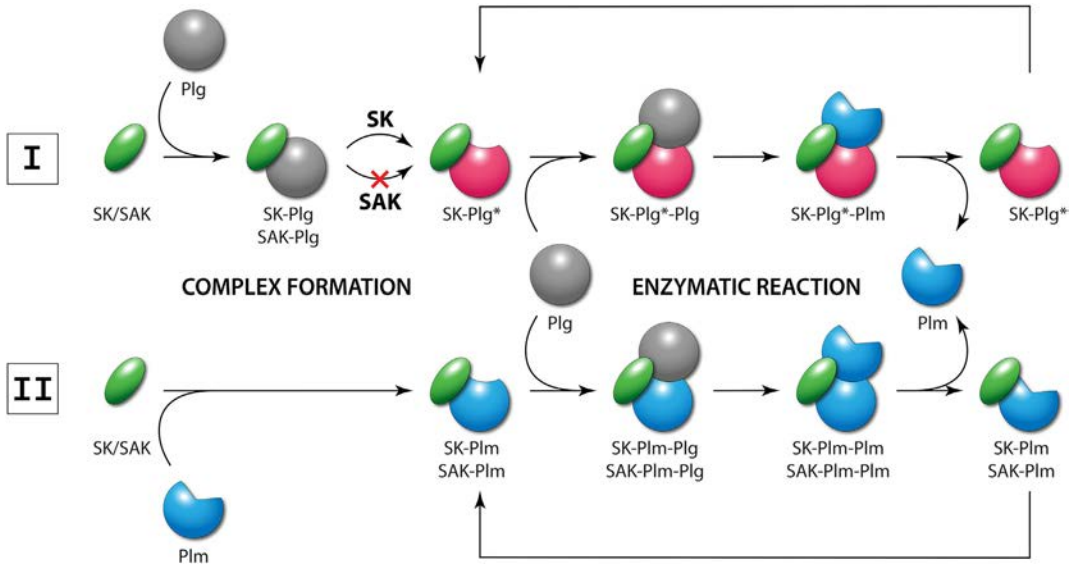

Fig. 13. Schematic representation of the indirect mechanism of plasminogen activation. *Pathway I*: Streptokinase (SK) or staphylokinase (SAK) form an equimolar complex with free plasminogen (Plg) but only SK is able to form an enzymatically active complex (SK-Plg*) with an open conformation of Plg. The activated complex then catalyzes the conversion of another free Plg molecule to active plasmin (Plm). *Pathway II*: An equimolar complex of SK/SAK with Plm is formed with higher affinity, does not require conformational activation and is enzymatically active with either SK or SAK. This complex (SK-Plm/SAK-Plm) catalyzes the same conversion of Plg to Plm as SK-Plg*. Compared to SK, SAK can activate Plg only via Pathway II.

5. When traces of plasmin molecules are generated, streptokinase tends to form the streptokinase-plasmin complex preferentially due to the approximately three orders of magnitude higher affinity towards plasmin compared to plasminogen. This complex does not require any conformational changes for activation as in the case of streptokinase-plasminogen but only narrows the broad substrate specificity of the plasmin partner towards the plasminogen substrate [199,219,221,225].

4.4. Binding and Stimulation by Fibrin

Due to the α domain, streptokinase does not require fibrin and activates plasminogen independently [186,194,200]. Stimulating experiments confirmed that neither fibrin nor fibrinogen had stimulatory effects on activation, preventing targeted thrombolysis on the surface of fibrin clots [185,186]. The activation independent of fibrin can lead to bleeding complications which represents one of the main drawbacks of the currently used streptokinase variant.

4.5. Inhibition

Unlike t-PA and urokinase, streptokinase has no natural inhibitor present in a human body. Since it is not homologous to eukaryotic plasminogen activators, it is not recognized by traditional plasminogen activator inhibitors [47]. Similarly, the streptokinase-plasminogen/streptokinase-plasmin complex is not inhibited by α_2-antiplasmin [226–228]. However, streptokinase's non-human origin causes the production of neutralizing antibodies by the human immune system when used as a drug. Thus, repeated administration decreases streptokinase's thrombolytic efficiency and can lead to a serious allergic response [188,189].

4.6. Protein Engineering

Different approaches were tested to enhance fibrin specificity of streptokinase. Deletion of the α domain prevented streptokinase to interact with the closed conformation of plasminogen. Instead, it formed the streptokinase-plasmin complex exclusively and was able to activate only the extended conformation of plasminogen bound to the fibrin surface. This step made streptokinase an activator resembling the molecule of staphylokinase [186,194,200,229]. An alternative strategy was based on using fibrin-specific streptokinases from different *Streptococcus* strains or on shuffling activators' domains to combine their properties. While keeping the original activity, the fibrin-specificity of these new molecules was comparable or even higher than for t-PA [185,230–232].

Different engineering attempts were focused on increasing the half-life of streptokinase. Strategies were based on finding that during the process of plasminogen activation, the generated plasmin non-physiologically cleaves streptokinase into three polypeptide chains (residues 1–59, 60–386, and 387–414) leading to drop in activity [233,234]. Preventing the cleavage by mutagenesis at the identified positions (Lys59Gln/Glu, Lys386Gln) resulted in a 21-fold increased half-life without affecting activity [235–237]. Alternative strategies based on combinatorial mutagenesis [14,238], PEGylation [239–242], glycosylation [235,243], or lipidification [244] yielded streptokinase variants with improved thrombolytic activity, decreased immunogenicity, and higher half-life.

A very successful variant of streptokinase is its acylated complexed form known as anistreplase. Anistreplase represents a pre-formed streptokinase-plasminogen complex with plasminogen's active site inactivated by anisoylation [245]. *In vivo*, p-anisic acid is cleaved and removed, resulting in an enzymatically active plasminogen activator. While keeping the original activity and antigenicity of streptokinase, the half-life of the variant was prolonged approximately 10-fold [246,247].

5. Staphylokinase

5.1. Biological Function

Staphylokinase is a small prokaryotic plasminogen activator secreted by lysogenic *Staphylococcus aureus*, enabling invasion of the host's tissue [248]. Originally mentioned already during the discovery of streptokinase in 1933 [182], the molecule of staphylokinase was firstly described in 1948 [248] and produced recombinantly in 1983 [249]. Although being immunogenic and having the half-life of only 6 min, its high fibrin specificity, low production cost, and promising clot penetrability make staphylokinase a potential thrombolytic for the clinical practice [47,250–253].

5.2. Molecular Structure

The protein has the smallest structure of all the biochemically characterized biological thrombolytics. It is composed of 136 amino acids, of which 30 % are charged [249,254,255]. The protein is folded into a single domain with the molecular weight of 15.5 kDa [256,257]. Structurally, but not sequentially, staphylokinase is homologous with the α domain of streptokinase with the β-grasp fold (Fig. 14) [256,258].

5.3. Mechanism of Activation and Specificity

Similarly to streptokinase, staphylokinase is an activator with an indirect mechanism of plasminogen activation, showing no enzymatic activity [248,259]. Compared to streptokinase, the complex of plasminogen bound by staphylokinase is inactive and is not able to perform the non-proteolytic activation of the plasminogen zymogen [260,261]. Instead, staphylokinase forms a productive equimolar (1:1) complex with plasmin only (Fig. 13 – Pathway II) [259,261,262]. Within the complex, staphylokinase modifies a broad substrate specificity of plasmin from a general protease digesting fibrin to an enzyme selectively cleaving the Arg561-Val562 bond in another molecule of plasminogen, yielding active plasmin [259]. Only a few studies have been conducted to understand staphylokinase at the molecular level. The results identified that the residues 26, 42–50, 65–69, and 75 are important for the plasmin "partner" binding [263–267], while the residues 11–16, 46–50, 65–69, and 97–98 are involved in the binding and the processing of the plasminogen "substrate" [256,266,268–270]. In contrast, the first ten

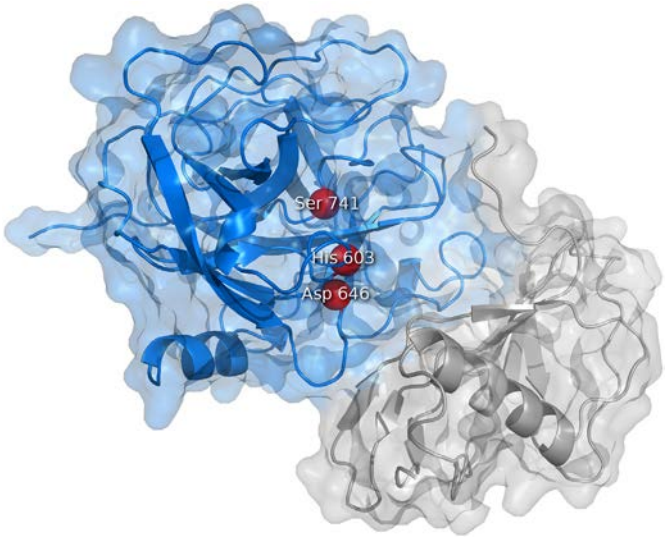

Fig. 14. The complex of staphylokinase with bound plasmin. The complex of staphylokinase (gray) and the catalytic domain of plasmin (blue) (PDB ID 1BUI). The catalytic triad of plasmin His603, Asp646, and Ser741 are shown as red beads.

N-terminal residues are not required for the activity and are cleaved by active plasmin [268,271–275].

5.4. Binding and Stimulation by Fibrin

An unusually high fibrin specificity of staphylokinase is given by mutual action of fibrin and α_2-antiplasmin. In the absence of fibrin, the staphylokinase-plasmin complex is rapidly inhibited by α_2-antiplasmin. In clotted plasma, fibrin competitively interacts with the complex via the same lysine binding sites as the inhibitor so the inhibition is prevented [226–228,276–278]. Such a mechanism allows staphylokinase to activate plasminogen preferentially only on the fibrin's surface. This avoids systemic plasminogen activation causing fibrinogenolysis and bleeding complications [226,228,253,276,277,279]. Moreover, the activity of staphylokinase is further enhanced by the fact that plasminogen/plasmin molecules bound to fibrin exhibit more extended conformation preferred by staphylokinase [278,280].

5.5. Inhibition

Compared to streptokinase, which is resistant to α_2-antiplasmin in any form, the staphylokinase-plasmin complex is rapidly inhibited in human plasma as described above [227,228,276,277,279]. Conveniently, staphylokinase can reversibly dissociate from the inhibited complex so its effective concentration in plasma is not decreased by the inhibition [281]. The main factor decreasing the efficiency of staphylokinase is, therefore, its immunogenicity causing the production of neutralizing antibodies [250].

5.6. Protein Engineering

The immunogenic property of staphylokinase prevents its repeated administration and represents its biggest disadvantage. Three non-overlapping immunodominant regions located on the protein surface represent the main epitopes recognized by the antibodies [282–285]. After disruption of the identified contacts by combinatorial mutagenesis, the immunogenicity of the best variant decreased to less than 30 % [283,286–289]. Other engineering strategies using PEGylation [290–295], glycosylation [296], protein fusion [288,297–301], and lipidification [302] provided variants of staphylokinase exhibiting higher efficiency, improved half-life, decreased immunogenicity, and a lower risk of reocclusion.

6. Desmoteplase

6.1. Biological Function

Desmoteplase is a plasminogen activator that was isolated from the saliva of the vampire bat *Desmodus rotundus* in 1974 [303]. It is employed by these hematophagous animals during feeding on the blood of livestock, maintaining blood fluidity and attenuating formation of clots. Thrombolytic properties of desmoteplase were observed already in 1932, but it took another 34 years to identify that this factor is an activator of plasminogen [304]. Thanks to its enormous fibrin specificity, an absence of neurotoxicity, and a highly prolonged half-life of more than 2 h, desmoteplase represents a promising activator with the potential for the treatment of cardiovascular diseases [116,305–309]. Although these beneficial properties were not observed during randomized clinical trials, further clinical tests are in progress [310,311].

6.2. Molecular Structure

The protein exhibits more than 72 % sequence homology with the human t-PA and analogically contains a finger F, an epidermal growth factor EGF, and a serine protease P domain (Fig. 15) but only one kringle K domain which is equivalent to kringle K1 in t-PA and lacks a lysine-binding site [312–314]. The structure is composed of 441 amino acids with the molecular weight of 52 kDa, contains three glycosylation sites Thr68, Asn117 and Asn362, and 14 disulfide bonds [312,313,315–317]. Desmoteplase does not possess any plasmin-sensitive cleavage site so it exists solely as a single chain form [314].

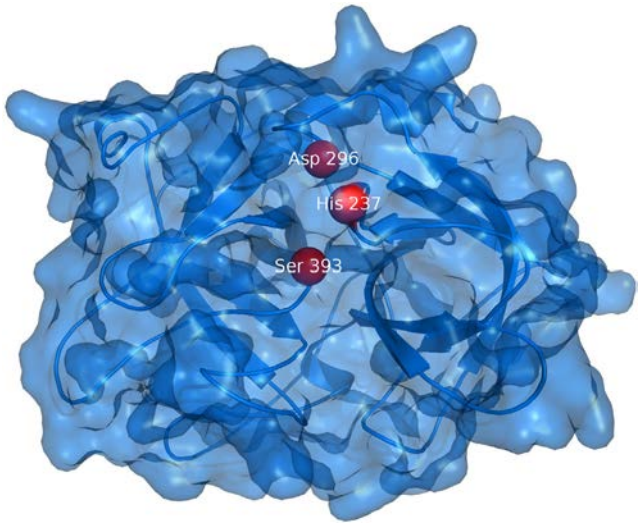

Fig. 15. The catalytic domain of desmoteplase. The catalytic domain of desmoteplase (PDB ID 1A5I) from the vampire bat *Desmodus rotundus* is shown as blue surface. The catalytic triad of His237, Asp296, and Ser393 is shown as red beads.

6.3. Mechanism of Activation and Specificity

Desmoteplase is a serine protease with the typical catalytic triad (His238-Asp297-Ser394) that cleaves the Arg561-Val562 peptide bond in plasminogen, yielding active plasmin [312–314]. However, the mechanism of the enormous desmoteplase specificity has not been explained yet and all the experimental data provide only indirect assumptions [312,314,318–320]. It is hypothesized that the activity is connected with the intramolecular interaction between Lys259 and Asp297. This interaction opens the active site cavity and is formed only when desmoteplase is bound to fibrin by the finger F domain. Dissociation from fibrin disrupts this interaction and leads to a reversible loss of activity [314,318,320]. Such a mechanism also corresponds to the exclusive single-chain form of desmoteplase because the side chain of its Lys259 substitutes the function of the new N-terminus important in two-chain forms of other eukaryotic activators [314].

6.4. Binding and Stimulation by Fibrin

Desmoteplase is highly specific towards fibrin and exhibits the highest stimulation of all known thrombolytics [312,315,318,321]. Desmoteplase is almost inactive towards plasminogen in the absence of fibrin but once bound, its activity reaches the level of stimulated t-PA. As a consequence, desmoteplase is stimulated by fibrin approximately 100,000-fold, which is 200-fold higher than described for t-PA [312,318,321]. Moreover, the presence of fibrinogen and other plasma factors induce desmoteplase's activity insignificantly so this activator is selective exclusively towards fibrin [318,319,321]. Such property prevents fibrinogen depletion and plasminemia which often cause side effects such as hemorrhage [305,306,308].

6.5. Inhibition

Despite desmoteplase's eukaryotic origin, its repeated administration caused a slight raise of neutralizing antibodies in animal models,

potentially decreasing the thrombolytic's efficiency [322]. Furthermore, the structure is recognized by the inhibitors PAI-1 and PAI-2 due to its similarity to t-PA and urokinase, yet the resistance to inhibition is relatively high [306].

6.6. Protein Engineering

Since desmoteplase exhibits plenty of positive properties and not many drawbacks, a little effort was put into its engineering. A chimeric protein combining the structures of desmoteplase and tenecteplase has been recently reported [132,323]. In this variant, the kringle 2 domain of tenecteplase was deleted and the finger domain was exchanged for the domain of desmoteplase with the aim to improve the protein's fibrin specificity. However, the constructed variant was only 8-fold more specific than t-PA but 25-fold less specific compared to desmoteplase [132]. Another approach was focused on preventing the creation of the two-chain form of t-PA. Substitution of the plasmin sensitive cleavage site with the corresponding sequence of desmoteplase (Arg275His, Ile276Ser, Lys277Thr) provided 28-fold higher fibrin specificity [318]. On the other hand, the reversed approach introducing the plasmin sensitive site (His191Arg, Ser192Ile, Thr193Lys) into desmoteplase led to the loss of specific activation which confirmed the importance of this region for the fibrin selectivity [318].

7. Perspectives

7.1. Novel Enzymes

Discovery of enzymes potentially serving as novel thrombolytics with interesting biological properties is a very attractive avenue for future research. Continuous advances in the next generation sequencing technologies provide a wealth of valuable information, which are stored in public genomic databases. These sequences can be systematically searched for novel biocatalysts by using bioinformatics tools and analyzed by computer modeling [324], cloned and experimentally characterized. Several enzymes directly cleaving fibrin, without plasminogen activation, have been recently described: (i) leech-derived Harobin, (ii) fibrinolytic enzyme from *Chlorella* algae and (iii) Nattokinase from soybeans [325–327]. The hunt for novel thrombolytics will result in the discovery of more potent enzymes with different mechanisms than the ones known to date.

7.2. Small Molecules

Small molecules from a marine fungus *Stachybotrys* such as staplabin bind and induce a conformational change in plasminogen, increasing its fibrin binding and susceptibility to activation by plasminogen activators [328]. Additional molecules are being isolated from different organisms or developed by chemical modification since the discovery of staplabin [329,330]. Their small size confers several advantages as easier production, lack of immunogenicity, anti-inflammatory effects and no cross-reactivity with other pathways [327]. Despite their small size, these molecules exhibit clot-targeted plasminogen activation [331] and current animal studies have shown promising results for example with the molecule SMTP-7 [332,333]. Isolation and chemical modification of novel biologically active small compounds is another promising trend in thrombolytics research.

7.3. Encapsulation & Targeted Release

Plasminogen activators' half-lives can be significantly enhanced by trapping them in liposomes and nanomaterials. Effectiveness of plasminogen activators can be improved by targeting their release using ultrasound, the presence of activated platelets, or shear-stress [334–338]. Encapsulation allows for the targeted release of both a neuroprotective and a thrombolytic agent [339]. Modification with covalently linked DNA or RNA allows disruption of interactions between t-PA and LRP1 receptor with concurrent regulation of enzymatic activity [340,341]. PEGylation can reduce immunogenicity and increase the half-life of prokaryotic plasminogen activators [239,292]. Newly established methods for targeted delivery will be used to target the action of plasminogen activators into the thrombus with minimized side-effects.

7.4. Adjuvants

Neurological side-effects such as neuroinflammation and disruption of the blood-brain barrier by the interaction of various molecules with t-PA and plasmin negatively influence the outcome of the therapy. Unfavorable interactions and oxidative stress on thrombolytics in ischemic conditions are being tackled by using antibodies, protein antagonists of receptor binding, or small molecules [342–348]. Anti-inflammatory cytokines were recently tested as adjuvant therapy to alleviate inflammatory side-effects [348,349]. Plasmin and plasminogen activators are inhibited quickly in the blood clot. Moreover, binding sites of plasminogen activators on fibrin are removed by tissue activable fibrinolysis inhibitor which also slows down fibrinolysis. These effects can be blocked via antibodies against PAI-1, α_2-antiplasmin, and tissue activatable fibrinolysis inhibitor, or by blocking two fibrinolytic inhibitors simultaneously by diabodies. This has proven to accelerate fibrinolysis *in vivo* [350–356]. PAI-1 can be also inhibited by small molecules such as tiplaxtinin or downregulated by statins and renin-angiotensin-aldosterone inhibitors [357]. The fibrinolytic system has complex connections to immune pathways, cerebrovascular coupling, nervous system, cell migration, metastasis, and cognition. These connections offer many targets for enhancing the efficacy of thrombolytics as well as their use for the treatment of non-thrombotic conditions.

7.5. Protein Engineering

Protein engineering studies of eukaryotic plasminogen activators are no longer aimed at increasing half-life since longer-circulating and inhibitor-resistant thrombolytics are available. One of the aims is reducing the side-effects via increasing fibrin specificity [358,359] or preventing interactions with the receptors [114,115,360]. Reducing side-effects also provides novel indications for thrombolytic therapy, such as drainage of intracerebral hemmorhages or extension of the time window in which thrombolytics can be administered [114,333]. Despite improvements in many key properties, e.g., fibrin selectivity, half-life, and inhibitor resistance; faster lysis of a thrombus is still of major concern. The structure-function relationships of fibrin-specific drugs need to be studied in a complex with fibrin, a large molecule of its own. The improvement in the structural techniques, such as small-angle X-ray scattering and cryo-EM, will allow solving even the large multi-protein complexes. Acquired structural data can be analyzed by various structural bioinformatics and computer modeling techniques that will bring insights for engineering activity, affinity, specificity and selectivity of thrombolytics.

7.6. Production Systems

Seventeen disulfide bridges of t-PA make this protein hard to express in prokaryotic systems. Even reteplase containing nine disulfide bridges have to be refolded after the purification in order to adopt an active conformation. Higher yields of properly folded t-PA were achieved via expression into the periplasm or co-expression with disulfide oxidoreductase [361,362]. Production in yeasts generally leads to higher yields and absence of endotoxin when compared to bacteria [363–365]. Other avenues are auto-induction, growth medium or cultivation protocol optimization [366,367], development of new refolding protocols [368–370] or new mammalian cell lines [371]. To avoid the demanding work with mammalian cell cultures, eukaryotic plasminogen activators can be produced in plants or extracted from the milk of transgenic animals

[372–376]. We envisage fine-tuning of available production systems and continuous search for the new expression and purification systems.

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Declarations of Competing Interests

The authors declare that they have no conflict of interest.

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